A New Survey Method for Citrus Tristeza Virus Disease Assessment

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ABSTRACT. Various citrus-producing states in the U.S. as well as the Caribbean, Central American, and South American countries have chosen to survey for severe citrus tristeza virus (CTV) strains to identify their location and level of infestation. Several survey methods have been used in the past but most have proven to provide estimates of incidence that are insufficiently accurate and precise for the purposes required. The assessment of CTV incidence by sampling involves laboratory testing by ELISA of plant material collected in the field. Using field data and computer simulation, methods of field data collection were compared. One method is similar to that used until recently by the Central California Tristeza Eradication Agency (CCTEA) program, in which about 4% of the trees in a planting block are sampled and the material from each tree sampled is assayed separately. This method is compared with an alternative method in which about 25% of the trees in a block are sampled, and material from groups of four trees is bulked and assayed together. The number of assays this new method requires is, therefore, equivalent to 6.25% of the tree population. Our comparative study indicated that the latter method results in increased accuracy and precision of estimates of citrus tristeza disease incidence without appreciable increase in the number of laboratory assays required. The new alternative method described here has recently been implemented by the CCTEA. An adaptation of this method is also presented to account for differences in spatial patterns of CTV when the brown citrus aphid is present or absent.

In many citrus-growing regions of the world, citrus is planted over very large areas. These plantings can either be contiguous or diffuse throughout the region in patchwork fashion. In either case, individual commercial plantings often consist of very large numbers of individual trees. Citrus tristeza virus (CTV) is often a concern for commercial citrus and examples exist that demonstrate its destructiveness to plantings in various locations around the world. The damage caused by CTV infection is dependent upon virus isolate, cultivar/rootstock combination, and isolate-cultivar/rootstock interaction. At times new citrus plantings or entire regions may be established with virus-free material. In other locations, the isolates that exist may not be particularly deleterious to the crop. In such cases, if a new, potentially damaging isolate of CTV is introduced into a citrus-growing area, eradication or suppression of the isolate may be desirable. Alternatively, growers in a region may decide to take a proactive approach and establish a policy to detect and eliminate new introductions quickly before they become established. For these reasons and others, an accurate and precise virus survey method is needed to detect low incidence CTV infestations.

One of the main problems that faces any individual or group that decides to survey for CTV is how to accomplish such a survey within large plantings or over extensive citrus regions with limited resources. In other words, sampling schemes for very large citrus-growing areas must be practical in terms of time, labor, and expense. For a CTV eradication program, a threshold of CTV incidence is often established, which, if exceeded, invokes a tree-by-tree census to determine the location of individual CTV-positive trees, which are subsequently

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removed. The key to the success of an eradication program is a sampling scheme that is capable of detecting CTV-infection at low incidence, and doing so relatively quickly and economically. This information is then evaluated to determine regulatory action.

Various regulatory agencies exist in citrus-growing areas around the world that regulate CTV to some extent. For example, the Central California Tristeza Eradication Agency (CCTEA) has been involved in sampling large portions of the California Central Valley for CTV in an effort to identify and eliminate infections. Until recently, the CCTEA has relied on a systematic sampling method that consisted of harvesting and testing leaf tissue from one out of every 25 trees in a grove to estimate virus incidence. In the present study we used the CCTEA method as a benchmark for the development of a new methodology. The CCTEA has adopted the new methods described in this paper.

Previous studies have demonstrated that the increase and spread of CTV can be characterized by two diverse pathosystems: i) where the melon or cotton aphid, *Aphis gossypii*, is the predominant species and the brown citrus aphid, *Toxoptera citricida*, is absent, and ii) where *T. citricida* is the predominant vector. In the CTV/*A. gossypii* pathosystem, spatial patterns of virus incidence are difficult to distinguish from random (2, 3). Thus, from a practical point of view, a random distribution of virus infection can be assumed. Conversely, virus distribution related to the CTV/*T. citricida* pathosystem has been demonstrated to be aggregated at several spatial scales (4, 5). To accurately survey and predict CTV incidence, the distribution pattern of the virus in the field must be taken into account by the sampling method.

Because CTV is not easily or accurately diagnosed visually especially if infections are asymptomatic or trees are grown on resistant rootstocks, researchers have come to rely on ELISA and other methods to detect CTV infection (1). The sensitivity of many CTV antibody assays is such that grouping of samples to a certain extent is possible without sacrificing detection sensitivity. The testing of material in groups provides a means of increasing the proportion of the population sampled without increasing to the same extent the number of tests required (9). The sampling methods proposed in this paper take advantage of group sampling to predict disease incidence at the single tree level.

The objectives of this study were to develop a new sampling methodology that can address either of the CTV pathosystems described above and to evaluate the accuracy and precision of this new sampling methodology. Accuracy and precision of estimates at low (<10%) incidence was a priority, as were the requirements for economical use of resources. The study raised a number of important methodological issues, including those concerned with: the characterization of the spatial pattern of infected plants; the estimation of CTV incidence at the individual tree scale after testing groups of trees; the determination of the appropriate number and size of groups of trees; and the validation of sampling schemes.

MATERIALS AND METHODS

Two sampling methods were examined and compared. The first was the systematic sampling method traditionally used by the CCTEA. In the case of the CCTEA scheme, the initial tree is selected at random from among any of the first five trees in the first five rows in the block to be sampled. Subsequently, every fifth tree in every fifth row is selected systematically. In practice, selection of a random starting posi-

tion has not been routinely used by the CCTEA. Rather, the first tree in the first row has been used as the initial starting position. For this method four or eight leaves are taken from each of the designated sample trees. The leaves from each tree are combined and processed via ELISA as a single sample. Thus, a total of 4% of the trees in the block are sampled and processed via ELISA.

The second sampling method tested, henceforth referred to as the hierarchical sampling (HS) method, is based on 'group testing'. For this method, a citrus planting is divided into groups of four trees, each group consisting of two trees in each of two adjacent rows in a rectangular pattern. One of the first four groups of trees is selected at random as a starting position, then every fourth group of trees is sampled (Fig. 1). Two leaves are taken from each of the four trees in the group, which are then combined into a bulk sample. The bulk sample is then processed via ELISA as a single sample. The HS method samples 25% of the trees in the block, but since four trees are assayed as a group, the number of individual ELISA tests is equivalent to 6.25% of the total number of trees in the block.

The HS method is based on the relationship between the number of diseased trees measured at two spatial scales, i.e., the CTV incidence of individual trees and the CTV incidence of groups of four trees (8, 9, 10). The number of CTV-positive trees in a block can be estimated by the number of CTV-positive groups of four trees by the following relationship:

$$\tilde{P}_{low} = 1 - (1 - \widehat{P}_{high})^{1/n} \qquad (1)$$

Where, n = the number of trees in the group which equals 4, $\widehat{P}_{high}$ is the proportion of CTV-positive groups, and $\tilde{P}_{low}$ is the estimated incidence of CTV-positive trees in the block (Fig. 2). The variance, $\widehat{v}_{low}$, associated with the above estimated of $\tilde{P}_{low}$ can be estimated from:

$$\widehat{v}_{low} = \frac{\widehat{P}_{high} \bullet (1 - \widehat{P}_{high})^{(2-n)/n}}{n^2} \qquad (2)$$

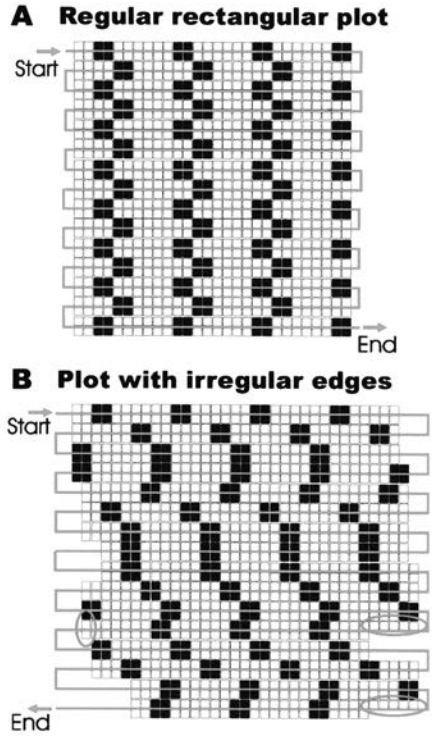

Fig. 1. Diagrammatic representation of the field implementation of the hierarchical sampling scheme. Position of individual trees is represented by black (sampled) or white (unsampled) squares. The gray line represents the path traversed by the field sampling team. The scheme requires the random selection of one of the first four groups of four trees as a starting point. In the examples above, the second group of four trees was randomly selected as a starting point. Leaf petioles are collected from each tree within a group and the collected materials are bulked. From this, the CTV status of the group is determined by ELISA. The incidence of CTV-positive groups is related to the incidence of CTV-positive trees, as described in the text by equation 1. A) Scheme for regular rectangular field. B) Scheme compensating for field with irregular boundaries.

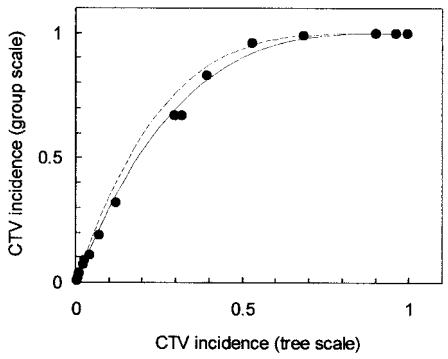

Fig. 2. The relationship between the incidence of CTV at the quadrat scale and its corresponding incidence at the single-tree scale. Data derived from disease assessments from CTV plots in northern Costa Rica are indicated by filled circles; the curve indicated by the dashed line is derived from the binomial distribution; the curve indicated by the solid line is derived from Equation 1.

For a full discussion of the development HS sampling method and the derivation of the equations above see Hughes and Gottwald (6). The above calculations have been demonstrated to hold true for the CTV/*A. gossypii* pathosystem in which the distribution of CTV-positive trees is indistinguishable from random (3). However, for the CTV/*T. citricida* pathosystem, where the distribution of CTV-positive trees is not random and aggregation can be detected at various spatial scales (4, 5), a slight modification of the above set of equations is necessary to ensure that the estimates do not overpredict incidence. This is accomplished by simply substituting n = 3.3 in both equations 1 and 2. For a full discussion of the modification to the HS method to account for spatial patterns of CTV resulting from spread of the virus due to *T. citricida*, see Hughes and Gottwald (7).

ELISA processing and sensitivity. Each composite sample was placed in 5 ml of PBS-Tween buffer and pulverized for 30 sec in a Kleco tissue pulverizer. Extracts were assayed for presence of CTV via double antibody sandwich indirect (DAS-I) ELISA (4).

The issue of assay sensitivity should also be addressed in relation to testing of material in groups. The laboratory assay must be sensitive enough to give a positive reaction if a single CTV-infected piece of tissue exists in the composite sample. An experiment was conducted to test the ability of the current ELISA method to detect various proportions of CTV-infected (isolate FS595) and healthy tissue in a composite sample. A dilution series consisting of 8:0, 7:1, 6:2, 5:3, 4:4, 3:5, 2:6, 1:7, and 0:8 (control) CTV-positive:CTV-negative leaf petioles were extracted in PBS-T at two-fold dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640 prepared in PBS-T. The coating antibody was IgG from a polyclonal antiserum No. 908 at 1 μg/ml. The intermediate antibody consisted of a mixture of two monoclonal antibodies, 11B1 and 3E10, which, in combination, act as a universal CTV probe capable of detecting all known CTV isolates (1). A sample was considered CTV-positive if the optical density (at 405 nm) of the reaction was greater than 0.10 above that of the control.

Testing of the hierarchical sampling scheme. Tests were conducted both by computer simulation and with actual field data. Simulation was used because insufficient field data existed that represented a large number of field plot distributions throughout a range of disease incidences. Simulated CTV incidence data were generated using a Microsoft Excel spreadsheet macro, *CTVSamplingSimulator* (T. R. Gottwald, unpublished). This simulator generated arrays (with user-defined numbers of rows and columns) in which a user-defined percentage of individual cells are designated '1' (CTV-positive) at random, the remaining cells being designated '0' (CTV-negative).

In order to validate the simulator, blocks of 400 trees, arranged 20 rows × 20 columns were generated. Ten blocks were generated for a range of CTV incidence consisting of 0.5 to 10.0% CTV-positive trees at 0.5% increments. The blocks were divided into quadrats of $n = 4$ trees, arranged two by two, and the number of quadrats in each block with zero, one, two, three or four CTV-positive trees was calculated (these operations are available as user-requested functions of the simulator). Thus, the frequency distribution of CTV-positive trees per quadrat was compiled for each simulated block. Both the observed variance and the corresponding binomial variance of each of these frequency distributions were calculated, as above, in preparation for analysis by the same method as was used for the field data.

Simulation study of sampling strategies. Two sampling schemes were compared.

A. CCTEA scheme based on sampling individual trees: The initial tree was selected at random from among the first five trees in each of the first five rows, then every fifth tree in every fifth row, systematically, after that. One of every 25 trees, or 4% of the population, was sampled.

B. HS scheme based on sampling groups of trees: A 20 × 20 block was considered to be 100 groups of four individual trees, each group arranged two by two. One of the first four groups was selected at random, then every fourth group systematically after that. Four of every 16 trees were sampled (25% of the population), but only CTV incidence at the group level (either no CTV-positive individuals versus at least one CTV-positive individual) was recorded.

Field tests. Data from 10 blocks of citrus, collected during surveys between 1992 and 1993 in California, were available from CCTEA records. These blocks varied in size between 304 and 512 trees. The location and CTV status of each tree had been recorded in the form of a map. CTV status of individual trees had been determined by ELISA, as either CTV-negative or CTV-positive. The range of CTV incidence among these blocks was 0.3 to 11.8%. For purposes of the study, the map of each block was divided into N quadrats (in this case, N varies from block to block) of $n = 4$ trees, arranged two rows by two trees along rows. The frequency distribution of CTV-positive trees per quadrat was then compiled for each block. The empirical (observed) variance and the corresponding theoretical (binomial) variance of each of these distributions were estimated. See Hughes and Gottwald for details (6).

For demonstrative purposes, the maps from the 10 California citrus blocks covering the range of incidence were also used as a basis for illustrating some characteristics of the CCTEA sampling scheme. For each selected map, all 25 possible estimates of incidence were calculated. This was achieved by systematically sampling every fifth tree in every fifth row, starting the sampling in turn from each of the 25 possible starting positions on each map.

Subsequent extension of the hierarchical sampling method. The above tests and simulations were conducted assuming the CTV/*A. gossypii* pathosystem in which virus distribution in the field is indistinguishable from a random pattern. The CTV/*T. citricida* pathosystem is more complex and characterized by virus incidence that occurs in nonrandom, i.e., loosely-aggregated, patterns. A simulation study was carried out in order to investigate the effect of CTV aggregation at the within-group scale on the estimation of CTV incidence at the scale of the individual tree from observations made at the group scale. The details of this simulation

TABLE 1
DEMONSTRATION OF THE ABILITY OF ELISA TO DETECT CTV IN MIXTURES OF CTV-POSITIVE AND CTV-NEGATIVE LEAF PETIOLES

Extract[z] dilution	CTV-positive: CTV-negative leaf petioles[y]								
	8:7	7:1	6:2	5:3	4:4	3:5	2:6	1:7	0:8
1:20	1.107	1.036	0.859	0.936	0.928	0.965	0.829	0.682	0.042
1:40	0.959	1.023	1.064	0.845	0.797	0.905	0.749	0.633	0.020
1:80	1.249	1.046	1.296	0.810	0.947	0.879	0.620	0.528	0.016
1:160	1.375	1.203	1.335	1.087	0.884	1.318	0.507	0.243	0.051
1:320	1.036	1.714	1.245	1.070	0.728	0.639	0.359	0.155	0.029
1:640	0.938	0.660	1.076	0.789	0.486	0.412	0.212	0.099	0.025

[z]Antiserum consisted of a mixture of two monoclonal antibodies, 11B1 and 3E10, which in combination act as a universal probe for CTV detection.
[y]Tissue was extracted in PBS-T. Values in the body of the table represent optical density at 405 nm. Values greater than twice that of the healthy control (0:8) for each antiserum dilution were considered CTV-positive.

study are rather complicated and the reader is referred to Hughes and Gottwald (7) for a complete description. Two simulation experiments were conducted. The first was intended to evaluate the performance of the simulator, the second was intended to test modifications to equations 1 and 2 as outlined above, as a means of obtaining estimates of CTV incidence for the CTV/*T. citricida* pathosystem at the scale of the individual tree from observations made at the group scale.

RESULTS AND DISCUSSION

ELISA sensitivity. The ability of ELISA to detect various mixtures of CTV-positive and CTV-negative tissue is shown in Table 1. The test demonstrated that the assay could easily detect a single CTV-infected petiole when mixed with seven healthy petioles. The extract dilution series further demonstrates that such discrimination is possible even at very low antiserum concentration, suggesting that CTV detection may be possible even when the ratio of CTV-positive:CTV-negative material is considerably less than 1:7.

Simulation results. Figure 3 shows the results of the simulation study of sampling strategies. For each sampling scheme and level of CTV incidence, the difference between CTV incidence estimated by sampling and the actual incidence were calculated separately for each of 100 replicate simulations. It is clear that the CCTEA sampling scheme was poorer in terms of both accuracy and precision compared to the HS method. Note that for the CCTEA scheme, the lower limit of the range coincides with the 25th percentile up to and including an actual CTV incidence of 9%. That is to say, even when the actual incidence is 9%, at least a quarter of the simulations of the CCTEA method found no CTV-positive trees among those sampled. Therefore, the HS scheme offers improvements over the CCTEA scheme in both accuracy and precision for very nearly the same level of laboratory effort

Adaptation of the HS method to the CTV/*T. citricida* pathosystem. Figure 4 demonstrates the results of the simulations studies that compare the use of Equations 1 and 2 with and without the adjustment in n that takes into account the differences in spatial aggregation between the CTV/*A. gossypii* and CTV/*T. citricida* pathosystems. The figures represent the difference between observed and actual CTV incidence over a range (0.0 to 0.2) of actual CTV incidence. Note the sec-

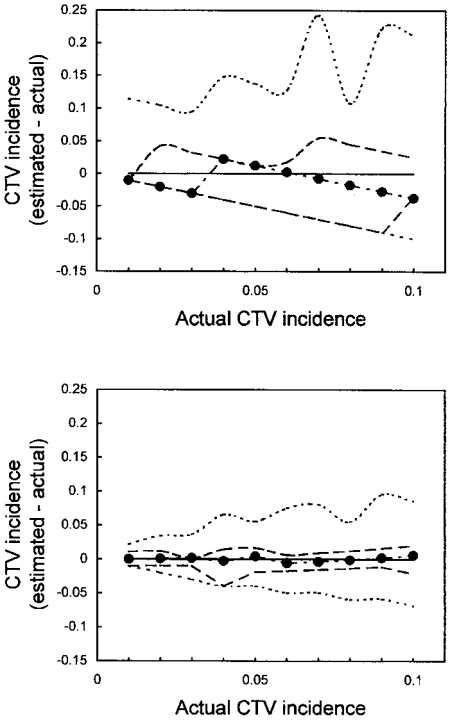

Fig. 3. Comparison of the CCTEA vs. HS sampling scheme. Figures represent the difference between observed and actual CTV incidence over a range (0.0 to 0.1) of actual CTV incidence. The top and bottom panels demonstrate, respectively, the incidence estimations resulting from the use of Equation 1 for the CCTEA and HS sampling schemes, respectively. The dotted intervals show the range covering all simulations (Lower dotted line in top panel is below the scale presented). The dashed intervals show the range covering the 25th to 75th percentiles. The dashed line joining the points shows the median value for the simulations. The solid horizontal line represents zero difference between observed and actual CTV incidence. Note the considerable improvement in mean estimations and reduced variance associated with the HS sampling scheme compared to the CCTEA scheme.

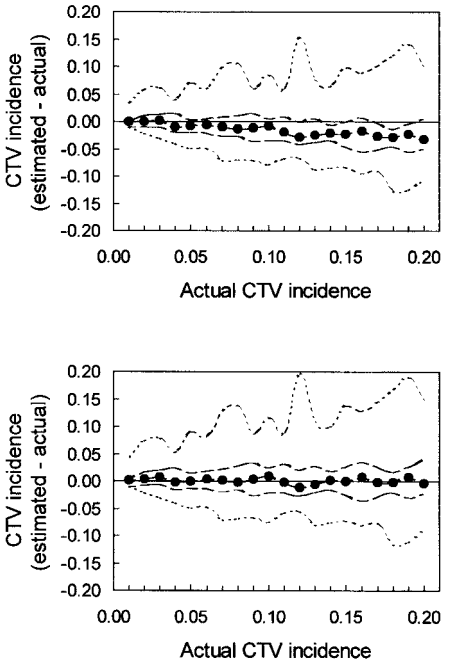

Fig. 4. Results of the simulation study of the HS sampling system when used to estimate the incidence of CTV for the CTV/*Toxoptera citricida* pathosystem. Figures represent the difference between observed and actual CTV incidence over a range (0.0 to 0.2) of actual CTV incidence. The top and bottom panels demonstrate, respectively, the corrected (n = 3.3) and uncorrected (n = 4) incidence estimations resulting from the use of Equation 1. The dotted intervals show the range covering all simulations. The dashed intervals show the range covering the 25th to 75th percentiles. The dashed line joining the points shows the median value for the simulations. The solid horizontal line represents zero difference between observed and actual CTV incidence. Note the second panel, representing use of the modified Equation 1, provides apparently unbiased estimates of CTV incidence.

ond panel representing estimates resulting from modification of Equation 1 appear to be unbiased. Without the slight modification to n, the original equation tended to slightly underestimate CTV incidence when applied to the CTV/*T. citricida* pathosystem.

Field tests. The use of simulation to study sampling schemes, and ultimately to validate a proposed scheme, allows a sampling scheme to be tested against known standards. In each case, the actual level and the pattern of disease are known, and the ability of different schemes to provide estimates of disease with acceptable levels of accu-

racy and precision can, therefore, be compared. Validation using field data is more difficult. Extensive resources are required to provide data sets against which sampling schemes can be tested and appropriate levels of replication in the range of disease incidence that is of interest cannot be guaranteed. The main purpose served by the collection of field data during the present study (Table 2) was, therefore, the refinement of field technique rather than the collection of a data set for a rigorous examination of the statistical properties of sampling schemes. Of the first ten blocks for which data are presented in Table 2, the estimate provided by the HS sampling scheme is closer to the actual CTV incidence in six cases, the estimate provided by the CCTEA sampling scheme is closer in two cases, and in the other two cases, no CTV-positive trees were detected by either sampling scheme. The remaining eight blocks in Table 2 provide field tests to compare the accuracy of the HS method to estimate CTV incidence when applied to either pathosystem.

The goal of this research study was to develop a sampling method to detect and estimate CTV incidence at low levels, i.e., trace to approximately 10 percent, primarily for regulatory purposes. When incidence exceeds the 10% threshold, the citrus blocks are usually placed

TABLE 2
ESTIMATES OF CTV INCIDENCE MADE FROM FIELD DATA

Block code[z]	Cultivar	Total number of trees	Actual CTV incidence[y]	Vector[x]	CCTEA scheme[w]		HS scheme[v]	
					$\widehat{P}$	s.e. ($\widehat{P}$)	$\tilde{P}$	s.e. ($\tilde{P}$)
(07)1-8	Naval	304	0.003	Ag	0	—	0	—
(07)35-46	Naval	456	0.015	Ag	0	—	0.018	0.013
(10A)25-32	Naval	513	0.019	Ag	0	—	0	—
(10B)34-41	Naval	512	0.033	Ag	0.036	0.041	0.060	0.022
(11A)9-16	Naval	512	0.041	Ag	0.036	0.041	0.040	0.018
(9B)43-50	Naval	512	0.049	Ag	0.071	0.058	0.024	0.014
(10A)1-8	Naval	495	0.065	Ag	0.042	0.046	0.054	0.022
(10B)42-49	Naval	512	0.076	Ag	0	—	0.033	0.016
(9A)9-16	Naval	512	0.096	Ag	0.143	0.078	0.111	0.030
(07)47-59	Naval	492	0.118	Ag	0.188	0.090	0.082	0.027
FL62-c2-34	Marsh	420	0.202	Ag			0.1856	0.0258
FL62-h2-34	RRVal	420	0.307	Ag			0.2479	0.0751
FL - B1	Tahiti Lime	400	0.283	Ag			0.4114	0.1588
CR4w	Pineapple	375	0.027	Tc			0.0117	0.0035
PR1	Val	266	0.109	Tc			0.1368	0.0423
PR2	Val	266	0.079	Tc			0.0823	0.0250
PR6	Val	266	0.120	Tc			0.1731	0.0542
DR3	Marsh	400	0.015	Tc			0.0246	0.0074

[z]The first 10 blocks in the table are used to compare the CCTEA vs. the HS sampling schemes. These blocks are arranged in order of increasing CTV incidence. The remaining eight blocks are provided to demonstrate the relative accuracy of the HS method against known CTV incidence.
[y]Calculated from the total number of CTV-positive trees (determined by ELISA) divided by the total number of trees in the block.
[x]The CTV pathosystem is defined by the predominant vector species, i.e., Ag = *Aphis gossypii*, or Tc = *Toxoptera citricida*.
[w]Calculated from data collected according to the systematic sampling scheme A. $\widehat{P}$ and $\tilde{P}$ are the estimated CTV incidences for the indicated planting via CCTEA and HS sampling schemes, respectively.
[v]Calculated from Equations 1 and 2 applied to data collected according to HS sampling scheme with appropriate adjustment for pathosystem.

in a different regulatory category relative to eradication or suppression. Although the HS method detected CTV in all cases relative to the second portion of Table 2, the reduced accuracy of the incidence estimates can be seen especially for situations for which CTV incidence exceeded 20%.

Implications of using increased bulking of samples in CTV detection. The potential of bulking tissue samples from numerous trees has often been proposed as a means of extending detection resources over large acreages. As described above, ELISA protocol can be extended to testing bulks of eight or more trees and still result in a positive test if only one sample has virus. Therefore, bulking tissue from four trees, as we have demonstrated for the HS method, is conservative and well within the capabilities of the assay method. However, with careful attention to ELISA protocol and use of some CTV antibodies, this could probably be extended further, perhaps to bulk samples consisting of a composite of samples from as many as 50 to 100 individual trees. It has been proposed that use of other detection methods such as PCR or other molecular assays could easily achieve this level of sensitivity or greater. However, there are statistical implications that need to be considered and may restrict or limit the number of individuals that can be bulked depending on the level of accuracy that is needed for a given survey, especially if the incidence is low.

For example, the proportion of false negative decisions that would arise if groves were classified as CTV-free on the basis of an assay of material from 100 trees combined into a single group is shown in Fig. 5. This shows, for example, that if the actual incidence of CTV infection is 2% (the threshold mentioned by Mathews et al. (11, 12), above which every tree in a grove would be tested), the probability of reaching a decision that the citrus block is CTV-free is around 13%. At actual incidence levels above 5%, the false negative proportion is negligible.

In the context of pathogen management decision-making, it is not only the sensitivity of the assay used for detection that is important, but also the characteristics of the sampling scheme in which the assay is deployed. Thus, the deployment of detection methods such as PCR that allow bulking of large numbers of individual samples to screening citrus blocks for presence or absence of CTV-infection, require careful consideration of the acceptable rate of false negative decisions in advance of the implementation of such a scheme. This would be particularly important if *T. citricida* were the main vector, since rates of increase from low to high incidence of CTV have been shown to be quicker with *T. citricida* than with *A. gossypii* (5). Thus a false negative assay of an infected block could result in a rapid increase and spread of undetected CTV in the area.

For CTV survey programs, the main objective is to obtain CTV incidence estimates that are both accurate and precise, particularly at low incidence. The main constraints are

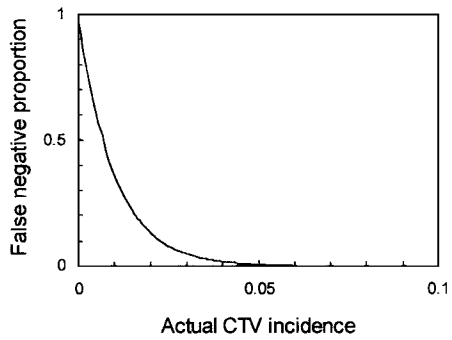

Fig. 5. The relationship between the proportion of false negative decisions and actual CTV incidence, if groves were classified as CTV-free on the basis of an assay of materials from 100 trees combined into a single group.

the costs and labor associated with field sample collection and laboratory ELISA tests. In the present study, the results of both simulation studies and field trials show, as may be expected, that as a larger proportion of the population is sampled, there is a corresponding increase in accuracy and precision of the estimate of incidence. Relative to the HS method, these improvements are preserved when the material from groups of trees is pooled and tested without distinguishing individual trees. Thus with the HS method, the proportion of the population sampled may be increased without having to increase appreciably to the same extent the number of ELISA tests required. In the present study, the groups (both actual and simulated) from which material were pooled comprised rectangular quadrats containing four trees. The benefits of the alternative sampling methodology based on this procedure are increased accuracy and precision of incidence estimates, with little additional field collection effort and only a small increase in the number of samples which undergo ELISA.

Sampling Software. An MS Excel spreadsheet is available which demonstrates the Hierarchical Sampling method, aids in the random selection of a starting position for field sample collection, and calculates CTV incidence for the CTV/*A. gossypii* and the CTV/*T. citricida* pathosystems as described above. For a copy of this spreadsheet, contact T. R. Gottwald at TGottwald@ushrl.ars.usda.gov.

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